NOVEL RUMEN BACTERIA VARIANTS AND PROCESS FOR PREPARING SUCCINIC ACID EMPLOYING THE SAME

TECHNICAL FIELD

The present invention relates to a rumen bacterial mutant which produce succinic acid at high concentration while producing little or no other organic acids, as well as a method for producing succinic acid, which is characterized by the culture of such mutants in anaerobic conditions.

BACKGROUND ART

Various anaerobic microorganisms, including *Succinivibrio dextrinosolvens*, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and the like, produce succinic acid as an end product by glucose metabolism (Zeikus, *Annu. Rev. Microbiol.*, 34:423, 1980). Strains that produce succinic acid at industrially useful yield have not yet been reported except for *Anaerobiospirillum succiniciproducens* known to produce succinic acid at high concentration and high yield from glucose upon the presence of excessive CO_2 (David *et al.*, *Int. J. Syst. Bacteriol.*, 26:498, 1976). However, since *Anaerobiospirillum succiniciproducens* is an obligate anaerobic microorganism, a fermentation process of producing succinic acid using this microorganism has a shortcoming that the process itself becomes unstable even upon exposure to a very small amount of oxygen.

To overcome this shortcoming, *Mannheimia succiniciproducens* 55E was developed that is a strain having not only resistance to oxygen but also high organic acid productivity. However, since this strain produces formic acid,

acetic acid and lactic acid in addition to succinic acid, it has shortcomings in that it has low yield and costs a great deal in a purification process of removing other organic acids except succinic acid.

Recombinant *E. coli* strains for the production of succinic acid have been reported in various literatures. If the *E. coli* strains have disruptions of a gene coding for lactate dehydrogenase and a gene coding for pyruvate formate-lyase, it is hard for them to grow in anaerobic conditions. Furthermore, they have too low yield to apply them to industrial field, since, although lactic acid is not produced as a fermentation product, other metabolites (acetic acid and ethanol) account for about half of the production of succinic acid. In an attempt to overcome such shortcomings, *E. coli* cells were grown in aerobic conditions, and then anaerobic conditions were applied to induce the fermentation of succinic acid. However, this attempt still has low productivity (Vemuri *et al.*, *J. Ind. Microbiol. Biotechnol.*, 28:325, 2002). Also, other examples were reported in which the genes of enzymes, such as pyruvate carboxylase, phosphoenolpyruvate carboxylase, phosphoenolpyruvate carboxykinase, and malic enzyme, that immobilize CO_2 in a metabolic pathway of succinic acid fermentation, are introduced into *E. coli*, thereby increasing the production of succinic acid (Vemuri *et al.*, *Appl. Environ. Microbiol.*, 68:1715, 2002; Millard *et al.*, *Appl. Environ. Microbiol.*, 62:1808, 1996; Chao and Liao, *Appl. Environ. Microbiol.*, 59:4261, 1993; Stols and Donnelly, *Appl. Environ. Microbiol.*, 63:2695, 1997).

Meanwhile, it is known that the disruption of *ptsG* in *E. coli* contributes to an improvement of bacterial production and succinic acid production (Chatterjee *et al.*, *Appl. Environ. Microbiol.*, 67:148. 2001), but most of rumen bacteria have no *ptsG*, and thus have an advantage that they do not require a removal process of *ptsG* as in the case of *E. coli*. Recently, an attempt is actively conducted in which the genes of enzymes that immobilize CO_2 in a metabolic pathway of succinic acid fermentation are introduced into rumen bacteria, including genus

Actinobacillus and genus *Anaerobiospirillum*. However, in this attempt, other organic acids were produced at large amounts or the yield of succinic acid was so low, as a result of that, it did not reach an industrially applicable level.

DISCLOSURE OF INVENTION

Accordingly, during our extensive studies to develop bacterial strains that produce succinic acid at high yield, the present inventors constructed bacterial mutant *Mannheimia* sp. LPK (KCTC 10558BP) by the disruption of a lactate dehydrogenase gene (*ldhA*) and a pyruvate formate-lyase gene (*pfl*) from *Mannheimia succiniciproducens* 55E, which is a kind of rumen bacteria, and constructed bacterial mutants *Mannheimia* sp. LPK7 and LPK4, by the disruption of phosphotransacetylase gene (*pta*) and an acetate kinase gene (*ackA*), and a phosphoenolpyruvate carboxylase gene (*ppc*), respectively from the LPK strain, and then confirmed that the culture of such bacterial mutants in anaerobic conditions provides succinic acid at high yield, thereby completing the present invention.

Therefore, a main object of the present invention is to provide a rumen bacterial mutant that produces succinic acid at high yield while producing no other organic acids, as well as a producing method thereof.

Another object of the present invention is to provide a method of producing succinic acid, which is characterized by the culture of the above bacterial mutants in anaerobic conditions.

To achieve the above objects, in one aspect, the present invention provides a rumen bacterial mutant which a lactate dehydrogenase-encoding gene (*ldhA*) and a pyruvate formate-lyase-encoding gene (*pfl*) have been disrupted, and has the

property of producing succinic acid at high concentration while producing little or no other organic acids in anaerobic conditions.

In another aspect, the present invention provides a rumen bacterial mutant which a lactate dehydrogenase-encoding gene (*ldhA*), a pyruvate formate-lyase-encoding gene (*pfl*), a phosphotransacetylase-encoding gene (*pta*) and a acetate kinase-encoding gene (*ackA*) have been disrupted, and has the property of producing succinic acid at high concentration while producing little or no other organic acids in anaerobic conditions.

In still another aspect, the present invention provides a rumen bacterial mutant which a lactate dehydrogenase-encoding gene (*ldhA*), a pyruvate formate-lyase-encoding gene (*pfl*), and a phosphoenolpyruvate carboxylase-encoding gene (*ppc*) have been disrupted, and has the property of producing succinic acid at high concentration while producing little or no other organic acids in anaerobic conditions.

In the present invention, the rumen bacteria are preferably homo-fermentative bacteria that may be selected from the group consisting of genus *Mannheimia*, genus *Actinobacillus* and genus *Anaerobiospirillum* and produce only succinic acid while producing little or no other organic acids. In a preferred embodiment of the present invention, the rumen bacterial mutant is *Mannheimia* sp. LPK, LPK7 or LPK4.

In still another aspect, the present invention provides a method for producing rumen bacterial mutant that has the property of producing succinic acid at high concentration while producing little or no other organic acids in anaerobic conditions, the method comprising disrupting a lactate dehydrogenase-encoding gene (*ldhA*) and a pyruvate formate-lyase-encoding gene (*pfl*) from rumen bacteria that are selected from the group consisting of genus *Mannheimia*, genus

Actinobacillus and genus *Anaerobiospirillum*.

In the inventive method for producing the rumen bacterial mutant, the disruptions of the *ldhA* and *pfl* genes are preferably performed by homologous recombination. The homologous recombination is preferably performed using a genetic exchange vector containing a disrupted *ldhA* and a genetic exchange vector containing a disrupted *pfl*. Preferably, the vector containing a disrupted *ldhA* is pMLKO-sacB, and the vector containing a disrupted *pfl* is pMPKO-sacB.

In yet another aspect, the present invention provides a method for producing rumen bacterial mutant that has the property of producing succinic acid at high concentration while producing little or no other organic acids in anaerobic conditions, the method comprising additionally disrupting a phosphotransacetylase-encoding gene (*pta*) and an acetate kinase-encoding gene (*ackA*) from rumen bacteria that are selected from the group consisting of genus *Mannheimia*, genus *Actinobacillus* and genus *Anaerobiospirillum*, and a lactate dehydrogenase-encoding gene (*ldhA*) and a pyruvate formate-lyase-encoding gene (*pfl*) have been disrupted.

The disruptions of the *pta* and *ackA* genes are preferably performed by homologous recombination. The homologous recombination is preferably performed using a genetic exchange vector containing a disrupted *pta* and *ackA*. The genetic exchange vector containing a disrupted *pta* and *ackA* is preferably pPTA-sacB.

In yet another aspect, the present invention provides a method for producing rumen bacterial mutant that has the property of producing succinic acid at high concentration while producing little or no other organic acids in anaerobic conditions, the method comprising additionally disrupting a phosphoenolpyruvate carboxylase-encoding gene (*ppc*) from rumen bacteria that are selected from the

group consisting of genus *Mannheimia*, genus *Actinobacillus* and genus *Anaerobiospirillum*, and a lactate dehydrogenase-encoding gene (*ldhA*) and a pyruvate formate-lyase-encoding gene (*pfl*) have been disrupted.

The disruption of the *ppc* gene is preferably performed by homologous recombination. The homologous recombination is preferably performed using a genetic exchange vector containing a disrupted *ppc*. The genetic exchange vector containing a disrupted *ppc* is preferably pPPC-sacB.

In the present invention, the rumen bacterial mutant having disruptions of a lactate dehydrogenase-encoding gene (*ldhA*) and a pyruvate formate-lyase-encoding gene (*pfl*) is preferably *Mannheimia* sp. LPK (KCTC 10558BP).

In yet another aspect, the present invention provides a genetic exchange vector pMLKO-sacB containing a disrupted *ldhA*; a genetic exchange vector pMPKO-sacB containing a disrupted *pfl*; a genetic exchange vector pPTA-sacB containing a disrupted *pta* and *ackA*; and a genetic exchange vector pPPC-sacB containing a disrupted *ppc*.

In another further aspect, the present invention provides a method for producing succinic acid, the method comprising the steps of: culturing the rumen bacterial mutants in anaerobic condition; and recovering succinic acid from the culture broth.

As used herein, the term "disruption" means that the genes encoding the enzymes are modified such that the enzymes cannot be produced.

In the present invention, each of the lactate dehydrogenase gene (*ldhA*) and the pyruvate formate-lyase gene (*pfl*) was identified from the genomic information of *Mannheimia succiniciproducens* 55E, which is a kind of rumen bacteria, and then,

all the two genes were removed from *Mannheimia succiniciproducens* 55E using a vector having disruptions of the genes, thereby constructing the bacterial mutant *Mannheimia* sp. LPK (KCTC 10558BP). Next, each of *pta-ackA* genes and a *ppc* gene was disrupted from the bacterial mutant *Mannheimia* sp. LPK, thereby constructing various bacterial mutants. Then, such bacterial mutants were confirmed to produce succinic acid at high concentration while producing little or no other organic acids.

The inventive bacterial mutants (*Mannheimia* sp. LPK, LPK4 and LPK7) are facultative anaerobic, gram-negative, non-mobile rods or cocobacilli, do not produce endospores, and can produce succinic acid in anaerobic conditions.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 shows a process of constructing a vector containing a disrupted *ldhA* (pMLKO-sacB).

FIG. 2 shows a process of constructing a vector containing a disrupted *pfl* (pMPKO-sacB).

FIG. 3 shows a process of constructing a bacterial mutant (LPK) by disrupting *ldhA* and *pfl* genes from *Mannheimia succiniciproducens* 55E.

FIG. 4 is an electrophoresis photograph showing the disruption of *ldhA* and *pfl* genes from *Mannheimia* sp. LPK (M: lambda *Hin*dIII size marker; lanes 1-3: PCR product LU1 & KM1 (1.5 kb); lanes 4-6: PCR product LD2 & KM2 (1.7 kb); lanes 7-9: PCR product PU1 & CM1 (2.2 kb); and lanes 10-12: PCR product PD2 & CM2 (1.6 kb)).

FIG. 5 shows the culture characteristics of *Mannheimia* sp. LPK in anaerobic conditions saturated with CO_2.

FIG. 6 shows a process of constructing vector containing a disrupted *pta* and *ackA* (pPTA-sacB).

FIG. 7 is a process of constructing a vector containing a disrupted *ppc* (pPPC-sacB).

FIG. 8 shows a process of constructing bacterial mutant LPK7 by disrupting *pta* and *ackA* genes from *Mannheimia* sp. LPK.

FIG. 9 shows a process of constructing bacterial mutant LPK4 by disrupting a *ppc* gene from *Mannheimia* sp. LPK.

FIG. 10 is an electrophoresis photograph showing the disruption of *pta* and *ackA* genes from *Mannheimia* sp. LPK7 (M: 1-kb ladder size marker; lane 1: PCR product P13 & P14 (1.1 kb); and lane 2: PCR product P15 & P16 (1.5 kb)).

FIG. 11 is an electrophoresis photograph showing the disruption of a *ppc* gene from *Mannheimia* sp. LPK4 (M: 1-kb ladder size marker; lane 1: PCR product P13 & P17 (1.1 kb); and lane 2: PCR product P15 & P18 (1.5 kb)).

FIG. 12 shows the cultivation characteristics of *Mannheimia* sp. LPK7 in anaerobic conditions saturated with CO_2.

FIG. 13 shows the cultivation characteristics of *Mannheimia* sp. LPK4 in anaerobic conditions saturated with CO_2.

DETAILED DESCRIPTION OF THE INVENTION

The present invention will hereinafter be described in further detail by examples. It will however be obvious to a person skilled in the art that these examples are given for illustrative purpose only, and the present invention is not limited to or by the examples.

Particularly, the following examples illustrate only a method comprising disrupting genes from a genus *Mannheimia* strain to obtain bacterial mutants and then producing succinic acid at high concentration by these bacterial mutants. However, methods by which bacterial mutants having disruptions of such genes are obtained from other rumen bacterial strains, such as genus *Actinobacillus* and genus *Anaerobiospirillum*, and succinic acid is produced using the bacterial strains, will also be obvious to a person skilled in the art.

Furthermore, the following examples illustrate only a certain medium and culture method. However, the use of other mediums different from, such as whey, corn steep liquor (CSL), as described in literatures (Lee *et al.*, *Bioprocess Biosyst. Eng.*, 26:63, 2003; Lee *et al.*, *Appl. Microbiol. Biotechnol.*, 58:663, 2002; Lee *et al.*, *Biotechnol. Lett.*, 25:111, 2003; Lee *et al.*, *Appl. Microbiol. Biotechnol.*, 54:23, 2000; Lee *et al.*, *Biotechnol. Bioeng.*, 72:41, 2001), and the use of various methods, such as fed-batch culture and continuous culture, will also be obvious to a person skilled in the art.

Example 1: Construction of pMLKO-sacB

In order to disrupt a lactate dehydrogenase gene (*ldhA*) by homologous recombination, a gene exchange vector was constructed in the following manner. First, the genomic DNA of *Mannheimia succiniciproducens* 55E (KCTC 0769BP), as a template, was subjected to PCR using primers set forth in SEQ ID NO: 1 and

SEQ ID NO: 2 below, and then, the obtained PCR fragment was cut with *Sac*I and *Pst*I and introduced into pUC18 (New England Biolabs, Inc., Beverly, Mass.), thereby constructing pUC18-L1.

SEQ ID NO: 1: 5'-CAGTGAAGGAGCTCCGTAACGCATCCGCCG (LS1)

SEQ ID NO: 2: 5'-CTTTATCGAATCTGCAGGCGGTTTCCAAAA (LP1)

Thereafter, the genomic DNA of *Mannheimia succiniciproducens* 55E, as a template, was subjected to PCR using primers set forth in SEQ ID NO: 3 and SEQ ID NO: 4 below, and the resulting PCR fragment was cut with *Pst*I and *Hind*III and introduced into the pUC18-L1, thereby constructing pUC18-L1-L2.

SEQ ID NO: 3: 5'-GTACTGTAAACTGCAGCTTTCATAGTTAGC (LP2)

SEQ ID NO: 4: 5'-GCCGAAAGTCAAGCTTGCCGTCGTTTAGTG (LH2)

pUC4K (Pharmacia, Freiburg, Germany) was cut with *Pst*I, and the resulting kanamycin-resistant gene was fused with pUC18-L1-L2 cut with *Pst*I, thereby constructing pUC18-L1-KmR-L2. A linker set forth in SEQ ID NO: 5 was inserted into the pUC18-L1-KmR-L2 cut with *Sac*I, thereby making a new *Xba*I cutting site.

SEQ ID NO: 5: 5'-TCTAGAAGCT

PCR on pKmobsacB (Schafer *et al.*, *Gene*, 145:69, 1994) as a template was performed using primers set forth in SEQ ID NO: 6 and 7 below, and the resulting PCR product was cut with *Xba*I and inserted into the above *Xba*I restriction enzyme site, thereby constructing pMLKO-sacB (FIG. 1).

SEQ ID NO: 6: 5'-GCTCTAGACCTTCTATCGCCTTCTTGACG (SXF)

SEQ ID NO: 7: 5'-GCTCTAGAGGCTACAAAATCACGGGCGTC (SXR)

Example 2: Construction of pMPKO-sacB

In order to disrupt a pyruvate formate-lyase gene (*pfl*) by homologous

recombination, a genetic exchange vector was constructed in the following manner. A pKmobsacB template containing a *sacB* gene (Genbank 02730) was subjected to PCR using primers set forth in SEQ ID NO: 8 and SEQ ID NO: 9 below. The resulting *sacB* product was cut with *Pst*I and *Bam*HI and inserted into pUC19 (Stratagene Cloning Systems. La Jolla, Calif.), thereby constructing pUC19-sacB.

SEQ ID NO: 8: 5'-AGCGGATCCCCTTCTATCGCCTTCTTGACG (SBG)

SEQ ID NO: 9: 5'-GTCCTGCAGGGCTACAAAATCACGGGCGTC (SPR)

The genomic DNA of *Mannheimia succiniciproducens* 55E, as a template, was subjected to PCR using primers set forth in SEQ ID NO: 10 and SEQ ID NO: 11 below. The resulting PCR fragment was cut with *Bam*HI and fused with the pUC19-sacB cut with *Bam*HI, thereby constructing pUC19-sacB-pfl.

SEQ ID NO: 10: 5'-CATGGCGGATCCAGGTACGCTGATTTCGAT (PB1)

SEQ ID NO: 11: 5'-CAAGGATCCAACGGATAAAGCTTTTATTAT (PB2)

In order to obtain a chloramphenicol-resistant gene, pACYC184 (New England Biolabs, Inc., Beverly, Mass.) as a template was subjected to PCR using primers set forth in SEQ ID NO: 12 and SEQ ID NO: 13 below. The resulting PCR product was cut with *Sma*I and fused with the pUC19-sacB-pfl cut with *Bst*1107I, thereby constructing pMPKO-sacB (FIG. 2).

SEQ ID NO: 12: 5'-CTCGAGCCCGGGGTTTAAGGGCACCAATAA (CTR)

SEQ ID NO: 13: 5'-CTCGAGCCCCGGGCTTTGCGCCGAATAAAT (CTF)

Example 3: Construction of *Mannheimia* sp. LPK strain

FIG. 3 shows a process of constructing a mutant strain (LPK) by disrupting *ldhA* and *pfl* genes from *Mannheimia succiniciproducens* 55E. *Mannheimia succiniciproducens* 55E was plated on LB-glucose medium containing 10 g/l of glucose, and cultured at 37°C for 36 hours. The colony formed was inoculated

in 10 ml of LB-glucose liquid medium, and cultured for 12 hours. The culture broth which had been sufficiently grown was inoculated by 1% in 100 ml of LB-glucose liquid medium, and cultured in a shaking incubator at 200 rpm and 37°C.

When the culture broth reached an OD of about 0.2-0.3 after 4~5hours, it was centrifuged at 4℃ and 4000 rpm for 10 minutes to collect cells. Then, the cells were resuspended in 200 ml of 10% glycerol solution at 4°C. The suspension was centrifuged at 4°C and 4000 rpm for 10 minutes, and the cells were collected and resuspended in 200 ml of 10% glycerol solution at 4°C, and then centrifuged at 4°C and 4000rpm for 10 minutes to collect the cells. The cells were suspended in glycerol at a volume ratio of 1:1, to obtain cell concentrate.

The cell concentrate thus obtained was mixed with the genetic exchange vectors pMLKO-sacB and pMPKO-sacB constructed in Examples 1 and 2, and then subjected to electroporation under conditions of 1.8 kV, 25 μF and 200 ohms. 1 ml of LB-glucose liquid medium was added to the electroporated mixture and cultured in a shaking incubator at 37°C and 200rpm for one hour. The culture broth was plated on LB-glucose solid medium containing a suitable antibiotic [Km (final concentration of 25 μg/ml) or Cm (6.8 μg/ml) and cultured at 37°C for 48 hours or more. In order to select a colony where only double crossover occurred, the colonies formed were streaked on LB-sucrose medium (LB medium with 100g/l sucrose) containing Km 25 μg/ml) or Cm (6.8μg/ml). After 24 hours, the formed colonies were streaked again on the same plate.

The colony (mutant) formed on the plate were cultured in LB-glucose liquid medium containing an antibiotic, and a genomic DNA was isolated from the cultured strain by the method described in Rochelle *et al. (FEMS Microbiol. Lett.*, 100:59, 1992). PCR was performed using the isolated mutant genomic DNA as a template, and the PCR product was electrophoresed to confirm the disruption of *ldhA* and *pfl* genes from the PCR product.

In order to confirm the disruption of the *ldhA* gene, PCRs were performed twice in the following manners. First, the mutant genomic DNA as a template was subjected to PCR using primers set forth in SEQ ID NO: 14 and SEQ ID NO: 15.

SEQ ID NO: 14: 5'-GACGTTTCCCGTTGAATATGGC (KM1)

SEQ ID NO: 15: 5'-CATTGAGGCGTATTATCAGGAAAC (LU1)

Then, the mutant genomic DNA as a template was subjected to PCR using primers set forth in SEQ ID NO: 16 and SEQ ID NO: 17 below. The products obtained in the two PCRs were subjected to gel electrophoresis to confirm the disruption of *ldhA* by their size (1.5 kb) (FIG. 4).

SEQ ID NO: 16: 5'-GCAGTTTCATTTGATGCTCGATG (KM2)

SEQ ID NO: 17: 5'-CCTCTTACGATGACGCATCTTTCC (LD2)

In order to confirm the disruption of *pfl*, PCRs were performed twice in the following manner. First, the mutant genomic DNA as a template was subjected to PCR using primers set forth in SEQ ID NO: 18 and SEQ ID NO: 19 below.

SEQ ID NO: 18: 5'-GGTGGTATATCCAGTGATTTTTTTCTCCAT (CM1)

SEQ ID NO: 19: 5'-CTTTGCAACATTATGGTATGTATTGCCG (PU1)

Then, the mutant genomic DNA as a template was subjected to PCR using primers set forth in SEQ ID NO: 20 and SEQ ID NO: 21. The products obtained in the two PCRs were subjected to gel electrophoresis to confirm the disruption of *pfl* by their size (1.5kb) (FIG. 4). In FIG. 4, M represents a Lambda *Hin*dIII size marker, lanes 1-3 represent the PCR product LU1 & KM1 (1.5kb), lanes 4-6 represent the PCR product LD2 & KM2 (1.7kb), lanes 7-9 represent the PCR product PU1 & CM1 (2.2kb), and lanes 10-12 represent the PCR product PD2 & CM2 (1.6kb).

SEQ ID NO: 20: 5'-TACTGCGATGAGTGGCAGGGCGGGGCGTAA (CM2)

SEQ ID NO: 21: 5'-CCCCAGCATGTGCAAATCTTCGTCAC (PD2)

The disruption of *ldhA* was confirmed by the fact that the product resulted from the PCR using the primers (LU1 and KM1) of SEQ ID NO: 14 and SEQ ID NO: 15 has a size of 1.5 kb an at the same time the product resulted from the PCR using the primers (LD2 and KM2) of SEQ ID NO: 16 and SEQ ID NO: 17 has a size of 1.7 kb. And, the disruption of *pfl* was confirmed by the fact that the product resulted from the PCR using the primers (PU1 and CM1) of SEQ ID NO: 18 and SEQ ID NO: 19 has a size of 2.2 kb and at the same time the product resulted from the PCR using the primers (PD2 and CM2) of SEQ ID NO: 20 and SEQ ID NO: 21 has a size of 1.6 kb. The position of each primer is shown in FIG. 3. The mutant constructed by the above method, i.e., a bacterial mutant having disruptions of *ldhA* and *pfl,* was named "*Mannheimia* sp. LPK" and deposited under accession number KCTC 10881BP on November 26, 2003 in the Korean Collection for Type Cultures (KCTC), Korean Research Institute of Bioscience and Biotechnology (KRIBB).

Example 4: Fermentation characteristics of *Mannheimia* sp. LPK

In order to examine the fermentation characteristics of *Mannheimia* sp. LPK constructed in Example 3 above, the mutant was cultured in anaerobic conditions saturated with CO_2, and the resulting reaction product was analyzed. First, carbon dioxide was introduced into 100 ml of preculture medium consisting of 20g/L glucose, 5g/L polypeptone, 5g/L yeast extract, 3g/L K_2HPO_4, 1g/L NaCl, 1g/L $(NH_4)_2SO_4$, 0.2g/L $CaCl_2 \cdot 2H_2O$, 0.2g/L $MgCl_2 \cdot 6H_2O$ and 10g/L $MgCO_3$, and then, *Mannheimia* sp. LPK was inoculated in the preculture medium and precultured at 39°C for 14 hours. Then, 0.9 L of culture medium consisting of 20g/L glucose, 5g/L polypeptone, 5g/L yeast extract, 3g/L K_2HPO_4, 1g/L NaCl, 5g/L $(NH_4)_2SO_4$, 0.2g/L $CaCl_2 \cdot 2H_2O$, 0.2g/L $MgCl_2 \cdot 6H_2O$ and 5g/L Na_2CO_3 was put in a 2.5-L culture tank, and 100 ml of the precultured microorganisms

were inoculated in the culture medium and batch-cultured at 39°C and pH 6.5 while supplying carbon dioxide at a flow rate of 0.25vvm.

The concentration of cells in the culture broth was measured with a spectrophotometer (Ultraspec 3000, Pharmacia Biotech., Sweden), and the amounts of succinate, glucose, lactate, acetate and formate were measured by HPLC (Aminex HPX-87H column, Bio-Rad, USA) (FIG. 5). Symbols in FIG. 5, refer to changes in the concentrations of cells (●), succinate (○), glucose (■), formate (◇) and acetate (△) with the passage of culture time. As shown in FIG. 5, after 14 hours of culture, the concentration of consumed glucose was 20g/L and the concentration of produced succinate was 17.2g/L, indicating that the yield of succinate (the amount of produced succinate/the amount of consumed glucose) is 81% and the volume productivity of succinate (the concentration of produced succinate/elapsed time) is 1.23 g/L/h. The inventive method of producing succinic acid by culturing *Mannheimia* sp. LPK in anaerobic conditions saturated with CO_2 showed a great increase in yield as compared to that of producing succinic acid by culturing parent strain *Mannheimia succiniciproducens* 55E in anaerobic conditions saturated with CO_2, and showed a ratio of succinic acid : acetic acid of 40.7:1, indicating that it can produce succinic acid with little or no by-products.

Example 5: Construction of pPTA-sacB

In order to disrupt a phosphotransacetylase gene (*pta*) and an acetate kinase gene (*ackA*) by homologous recombination, a genetic exchange vector was constructed in the following manner. First, the genomic DNA of *Mannheimia* sp. LPK (KCTC 10558BP), as a template, was amplified by PCR using primers set forth in SEQ ID NO: 22 and SEQ ID NO: 23 below, and the resulting PCR fragment was cut with *Xba*I and *Bam*HI and introduced into pUC19, thereby constructing pUC19-PTA1.

SEQ ID NO: 22: 5'-GCTCTAGATATCCGCAGTATCACTTTCTGCGC

SEQ ID NO: 23: 5'-TCCGCAGTCGGATCCGGGTTAACCGCACAG

Thereafter, the genomic DNA of *Mannheimia* sp. LPK as a template was amplified by PCR using primers set forth in SEQ ID NO: 24 and SEQ ID NO: 25 below, and the resulting PCR fragment was cut with *Xba*I and *Sac*I and introduced into the pUC19-PTA1, thereby constructing pUC19-PTA12.

SEQ ID NO: 24: 5'-GGGGAGCTCGCTAACTTAGCTTCTAAAGGCCATGT TTCC

SEQ ID NO: 25: 5'-GCTCTAGATATCCGGGTCAATATCGCCGCAAC

As a template, plasmid pIC156 (Steinmetz *et al.*, *Gene*, 142:79, 1994) containing a spectinomycin-resistant gene (GenBank X02588) was amplified by PCR using primers set forth in SEQ ID NO: 26 and SEQ ID NO: 27 below, and the resulting PCR fragment (spectinomycin-resistant gene) was cut with *Eco*RV and introduced into the pUC19-PTA12, thereby constructing pUC19-PTA1S2 having the spectinomycin-resistant gene. The constructed pUC19-PTA1S2 was cut with *Sac*I and *Bam*HI and introduced into pUC19-SacB (see Example 2), thereby constructing a pPTA-sacB vector (FIG. 6).

SEQ ID NO: 26: 5'-GAATTCGAGCTCGCCCGGGGATCGATCCTC

SEQ ID NO: 27: 5'-CCCGGGCCGACAGGCTTTGAAGCATGCAAATGTCAC

Example 6: Construction of pPPC-sacB

In order to disrupt a phosphoenolpyruvate carboxylase gene (*ppc*) by homologous recombination, a genetic exchange vector was constructed in the following manner. First, the genomic DNA of *Mannheimia* sp. LPK, as a template, was amplified by PCR using primers set forth in SEQ ID NO: 28 and SEQ ID NO: 29, and the resulting PCR fragment was cut with *Xba*I and *Bam*HI and introduced into pUC19, thereby constructing pUC19-PPC1.

SEQ ID NO: 28: 5'-TACGGATCCCCAGAAAATCGCCCCCATGCCGA

SEQ ID NO: 29: 5'-GCTCTAGATATCGTTTGATATTGTTCCGCCACATTTG

Thereafter, the genomic DNA of *Mannheimia* sp. LPK, as a template, was subjected to PCR using primers set forth in SEQ ID NO: 30 and SEQ ID NO: 31, and the resulting PCR fragment was cut with *Xba*I and *Sac*I and introduced into the pUC19-PPC1, thereby constructing pUC19-PPC12.

SEQ ID NO: 30: 5'-GCTCTAGATATCCGTCAGGAAAGCACCCGCCATAGC

SEQ ID NO: 31: 5'-GGGGAGCTCGTGTGGCGCTGCGGAAGTAAGGCAAAAATC

A spectinomycin-resistant gene cut with *Eco*RV (see Example 5) was introduced into the pUC19-PPC12 to construct pUC19-PPC1S2. The pUC19-PPC1S2 was cut with *Sac*I and *Bam*HI and introduced into the pUC19-SacB, thereby constructing a pPPC-sacB vector (FIG. 7).

Example 7: Construction of *Mannheimia* sp. LPK7 and LPK4 strains

FIG. 8 and FIG. 9 show processes of constructing mutant strains LPK7 and LPK4 by disrupting *pta-ackA* and *ppc* from *Mannheimia* sp. LPK, respectively. *Mannheimia* sp. LPK was plated on LB-glucose medium containing 10g/l glucose, and cultured at 37°C for 36 hours. The colony formed was inoculated in 10 ml LB-glucose liquid medium and cultured for 12 hours. The culture broth which had been sufficiently grown was inoculated by 1% in 100 ml LB-glucose liquid medium and cultured in a shaking incubator at 37°C.

Cell concentrate was collected from the resulting culture broth in the same manner as described in Example 3. The collected cell concentrate was mixed with the genetic exchange vectors pPTA-sacB and pPPC-sacB constructed in Examples 5 and 6, and then subjected to electroporation under conditions of 1.8 kV, 25°F and 200 ohms. The electroporated mixture was added with 1 ml of

LB-glucose liquid medium and cultured in a shaking incubator at 200 rpm and 37°C for one hour.

The culture broth was plated on LB-glucose solid medium containing a spectinomycin antibiotic (final concentration: 50 (g/ml), and cultured at 37°C for at least 48 hours. In order to select a colony where double crossover occurred, the colonies formed were streaked on LB-sucrose medium (LB medium containing 100 g/l of sucrose) containing 50 (g/ml of spectinomycin. After 24 hours, the formed colonies were re-streaked on the same plate. The colony (mutant) formed on the plate was cultured in LB-glucose liquid medium containing an antibiotic, and a genomic DNA was isolated from the cultured strain by the method of Rochelle et al. The isolated mutant genomic DNA as a template was amplified by PCR, and the PCR product was electrophoresed to confirm the disruption of each of *pta-ackA* and *ppc*.

To confirm the disruption of *pta-ackA*, PCRs were performed twice in the following manner. First, the mutant genomic DNA as a template was subjected to PCR using primers set forth in SEQ ID NO: 32 and SEQ ID NO: 33 below. Then, the mutant genomic DNA as a template was subjected to PCR using primers set forth in SEQ ID NO: 34 and SEQ ID NO: 35.

SEQ ID NO: 32: 5'-CCTGCAGGCATGCAAGCTTGGGCTGCAGGTCGACTC

SEQ ID NO: 33: 5'-GCTGCCAAACAACCGAAAATACCGCAATAAACGGC

SEQ ID NO: 34: 5'-GCATGTAACTTTACTGGATATAGCTAGAAAAGGCATCGGGGAG

SEQ ID NO: 35: 5'-GCAACGCGAGGGTCAATACCGAAGGATTTCGCCG

The products obtained in the two PCRs were subjected to gel electrophoresis to confirm the disruption of *pta-ackA* by their size (FIG. 10). In FIG. 10, M represents a 1-kb ladder size marker, lane 1 represents the PCR product P13 & P14 (1.1 kb), and lane 2 represents the PCR product P15 & P16 (1.5 kb). The disruption of *pta-ackA* was confirmed by the fact the product resulted from the

PCR using the primers of SEQ ID NO: 32 and SEQ ID NO: 33 (P13 & P14) has a size of 1.1 kb at the same time the product resulted from the PCR using the primers of SEQ ID NO: 34 and SEQ ID NO: 35 (P15 & P16) has a size of 1.5 kb. The positions of the primers are shown in FIG. 8. The mutant strain constructed as described above, i.e., a strain resulted from the disruption of *pta-ackA* from *Mannheimia* sp. LPK, was named "*Mannheimia* sp. LPK7" and deposited under accession number "KCTC 10626BP" in KCTC, an international depositary authority.

Furthermore, to confirm the disruption of *ppc*, PCRs were performed twice in the following manner. First, the mutant genomic DNA as a template was subjected to PCR using primers set forth in SEQ ID NO: 32 and SEQ ID NPO: 36. Then, the mutant genomic DNA as a template was subjected to PCR using primers set forth in SEQ ID NO: 34 and SEQ ID NO: 37.

SEQ ID NO: 36: 5'-GATCCAGGGAATGGCACGCAGGCTTTCAACGCCGCC

SEQ ID NO: 37: 5'-GCAAAGCCAGAGGAATGGATGCCATTAACCAATAGCG

The products obtained in the two PCRs were subjected to gel electrophoresis to confirm the disruption of *ppc* by their size (FIG. 11). In FIG. 11, M represents a 1-kb ladder size marker, lane 1 is the PCR product P13 & P17 (1.1kb), and lane 2 represents the PCR product P15 & P18 (1.5kb). The disruption of *ppc* was confirmed by the fact that the product resulted from the PCR using the primers of SEQ ID NO: 32 and SEQ ID NO: 36 (P13 & P17) has a size of 1.1 kb at the same time the product resulted from the PCR using the primers of SEQ ID NO: 34 and SEQ ID NO: 37 (P15 & P18) has a size of 1.5 kb. The positions of the primers are shown in FIG. 9. The mutant strain constructed as described above, i.e., a strain resulted from the disruption of *ppc* from *Mannheimia* sp. LPK, was named "*Mannheimia* sp. LPK4".

Example 8: Fermentation characteristics of LPK7 and LPK4

In order to examine the fermentation characteristics of *Mannheimia* sp. LPK7 and LPK4 constructed in Example 7 above, the mutant strains were cultured in anaerobic conditions saturated with CO_2, and the resulting reaction products were analyzed. First, carbon dioxide was introduced into 200ml of the preculture medium as described in Example 4, and each of *Mannheimia* sp. LPK7 and LPK4 was inoculated in the preculture medium and precultured at 39°C for 24 hours. Next, 1.8 L of a culture medium, which is the same as that in Example 4 except that glucose concentration is 18 g/L (final 100mM), was put in a 6.6 L culture tank, and 100 ml of the precultured microorganisms was inoculated in the culture medium and then batch-cultured at 39°C and pH 6.5 while supplying carbon dioxide at a flow rate of 0.25vvm.

The concentrations of cells, succinate, glucose, lactate, acetate and formate were measured in the same manner as in Example 4 (FIG. 12 and FIG. 13). Symbols in FIG. 12 and FIG. 13 refer to changes in the concentrations of cells (● in upper portion), succinate (● in lower portion), glucose (□), formate (◆) and acetate (▲) with the passage of culture time. As shown in FIG. 12, after 22 hours of the culture of *Mannheimia* sp. LPK7, the concentration of consumed glucose was 100mM and the concentration of produced succinate was 124mM, indicating that the yield of succinate (the amount of produced succinate/the amount of consumed glucose) is 124 mol%. And, the production of acetate was remarkably reduced (Table 1). Also, as shown in FIG. 13, after 22 hours of the culture of *Mannheimia* sp. LPK4, the concentration of consumed glucose was 100mM and the concentration of produced succinate was 123.7mM, indicating that the yield of succinate (the amount of produced succinate/the amount of consumed glucose) is 123.7 mol%. And, the production of acetate was greatly reduced as compared to that in the wild type (Table 1).

The inventive method of producing succinic acid by culturing *Mannheimia* sp. LPK7 in anaerobic conditions saturated with CO_2 showed a great increase in the yield of succinic acid and also a 9.8 times increase in the ratio of succinic acid: acetic acid, as compared to that of producing succinic acid by culturing parent strain *Mannheimia succiniciproducens* 55E in anaerobic conditions saturated with CO_2, indicating that the inventive method can produce succinic acid with producing little or no byproducts (Table 1).

As reported by Bulter et al., even if acetate-producing genes in microorganisms known till now are all disrupted, a significant amount of acetate is produced in amino acid and fatty acid metabolisms which are still not established (Bulter *et al. PNAS*, 101:2299, 2004). Thus, the present invention cut off all acetate production pathways known till now, and achieved succinate fermentation at high yield and concentration.

Table 1: Comparison of products from fermentation of LPK4 and LPK7 and product from fermentation of 55E in anaerobic conditions

Strain	Fermentation products (mM)						S/A ratio (fold)
	Succinate	Acetate	Formate	Lactate	Pyruvate	Ethanol	
55E	99.1	40.6	53.8	8.2	13	<1.0	2.44 (1.0)
LPK4	123.7±6.2	28.1±5.4	ND	ND	12.2±6.3	<1.0	4.40 (1.8)
LPK7	124.0±5.2	5.2±0.2	ND	ND	36.36±4.7	<1.0	23.84 (9.8)

While the present invention has been described in detail with reference to the specific features, it will be apparent to persons skilled in the art that this description is only for a preferred embodiment and does not limit the scope of the present invention. Thus, the substantial scope of the present invention will be defined by the appended claims and equivalents thereof.

INDUSTRIAL APPLICABILITY

As described and provided above in detail, *Mannheimia* sp. mutant strains (LPK, LPK7 and LPK4) produce succinic acid in anaerobic conditions saturated with CO_2 and are facultative anaerobic strains having high resistance to oxygen. Thus, the production of succinic acid using such mutants can not only eliminate the fermentation process instability caused by oxygen exposure, etc., but also eliminate the production of other organic acids, as compared to the prior method of producing succinic acid using obligate anaerobic strains, thereby making it possible to optimize and maximize a purification process and production yield.

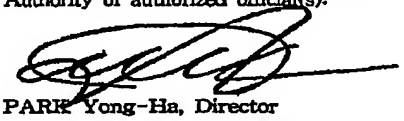
BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO : LEE, Sang Yup
Korea Advanced Institute of Science and Technology,
#373-1, Kuseong-dong, Yuseong-gu, Daejeon 305-701,
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: ***Mannheimia* sp. LPK**	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: **KCTC 10553BP**
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: [x] a scientific description [ ] a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **November 26 2003.**	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: **Korean Collection for Type Cultures** Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s):  PARK Yong-Ha, Director Date: **November 28 2003**

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BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

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Korea Advanced Institute of Science and Technology,
#373-1, Kuseong-dong, Yuseong-ku, Daejeon 305-701,
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
***Mannheimia* sp. LPK7**	**KCTC 10626BP**

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:
[x] a scientific description
[ ] a proposed taxonomic designation
(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **April 22 2004.**

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V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: **Korean Collection for Type Cultures**	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized officials):
Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	PARK, Yong-Ha Director Date: **April 27 2004**

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